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Communication

The Protease Inhibitory Properties of the Alzheimer's β-Amyloid Precursor Protein*

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We have expressed the 57-amino acid Kunitz domain of the Alzheimer's β-amyloid precursor protein (APP751) as a bacterial fusion protein. The protease inhibitory properties of the purified fusion protein, BX9, were virtually identical in all respects tested to those of purified secreted APP751. Both proteins strongly inhibited pancreatic trypsin (K_is = 0.2 and 0.3 nM) and less well epidermal growth factor-binding protein (K_is = 1 and 3.5 nM), α-chymotrypsin (K_is = 3 and 6 nM), and the γ-subunit of nerve growth factor (K_is = 8 and 9 M). Neither protein appreciably inhibited plasma and pancreatic kallikreins, thrombin, lung tryptase, neutrophil elastase, or cathepsin G. The remarkable similarity of the protease inhibitory profile of BX9 to that of secreted APP751 suggests that proper intramolecular disulfide bond formation has occurred in the bacterial fusion protein and leads to the conclusion that the amyloid precursor protein Kunitz domain is a relatively specific inhibitor of only a few trypsin-like arginine esterases.

The β-amyloid precursor protein (APP)[1] in Alzheimer's disease is a membrane-spanning protein (Dyrks *et al.*, 1988), two forms of which, APP751 and APP770, contain a 57-amino acid insert with striking homology to the Kunitz family of protease inhibitors (Tanzi *et al.*, 1988; Ponte *et al.*, 1988; Kitaguchi *et al.*, 1988). We have previously shown (Oltersdorf *et al.*, 1989) that the secreted form of APP751 (sAPP751) with this Kunitz protease inhibitor domain is protease nexin-II, which was first isolated from human fibroblasts (Knauer and Cunningham, 1982) by its property of forming complexes with epidermal growth factor-binding protein (EGF-BP), a serine esterase from mouse submaxillary glands. To better study the protease inhibitory properties of the APPs with the Kunitz domain insert, purified amounts of which were available only in limited quantities, we expressed the Kunitz domain as a fusion protein in a temperature-inducible *Escherichia coli* espression system. Somewhat unexpectedly, the fusion protein was active as a trypsin inhibitor, without benefit of further refolding, when assayed in a crude partially purified form. In this communication we report that the inhibitory properties of the purified fusion protein, designated BX9, very closely matched that of purified sAPP751 and document the inhibition constants obtained with the two proteins toward a number of serine proteases.

EXPERIMENTAL PROCEDURES

Materials—APP751 cDNA was a gift from Dr. Rudi Tanzi, Massachusetts General Hospital. Trypsin, α-chymotrypsin, and plasmin were obtained from Sigma. Pancreatic and tissue kallikreins were Boehringer-Mannheim products. Neutrophil elastase, cathepsin G, and lung tryptase were a gift from Dr. James Travis, University of Georgia. EGF-BP was purified from mouse submaxillary glands by M. B. and R. A. B. as previously described (Blaber *et al.*, 1989), and mouse glandular kallikrein (mGK-22) was purified by M. A. and W. C. M. as described earlier (Wilson and Shooter, 1979). γ-NGF was purified from 7 S NGF (Collaborative Research). Synthetic substrates were purchased from Sigma, Helena Laboratories, and Bachem.

Cloning and Expression of Kunitz Domain Fusion Protein—A 218-base pair *TaqI* restriction fragment of APP cDNA containing the Kunitz protease inhibitor domain was cloned into a modified version of the expression vector pEX10mer, utilizing the heat-inducible bacteriophage λ P_L promoter (Seedorf *et al.*, 1987). The expression product is a 194-residue fusion protein, the predicted sequence of which is shown in Fig. 1. After induction of the protein in *E. coli* strain N6405, the cells were sonicated in 0.1% Nonidet P-40 and 0.5% sodium deoxycholate, and the insoluble pellet was extracted in 7 M urea. The fusion protein was recovered in the supernatant after centrifugation.

Purification of the Fusion Protein—The 7 M urea extract was acidified, centrifuged, and the supernatant pumped directly onto a 1 × 25-cm C_4 (Vydac) reverse-phase column. Bound protein was eluted with a linear gradient of 0.1% trifluoroacetic acid/acetonitrile to 60%. Fractions collected were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and for activity by trypsin inhibition assay (see "Assay of Protease Inhibition"). Peak-purified fractions were pooled and then lyophilized.

Purification of Secreted APP751—The secreted form of APP751 was purified from the conditioned medium of transfected 293 cells as described earlier (Oltersdorf *et al.*, 1989).

Assay of Protease Inhibition—All assays were done in 0.1 M Tris-HCl, pH 7.5, with 0.5 M NaCl, 0.1% gelatin, and 0.05% Triton X-100. Protease (5 to 50 nM) was incubated with increasing concentrations of inhibitor protein (usually to 5 × protease concentration) in polypropylene microcentrifuge tubes for 10 min at 25 °C. 200-μl aliquots were then pipetted into 96-well microtiter plates, and the appropriate synthetic substrate was added to 0.25 mM. Initial rates at 25 °C were recorded by the SOFTMAX kinetic program on a Molecular Devices V_{max} kinetic microplate reader. The concentrations of active trypsin and α-chymotrypsin were determined by active site titration (Chase and Shaw, 1967; Bender *et al.*, 1966). The concentrations of EGF-BP, γ-NGF, and mGK22 were estimated from their known extinction coefficients (Blaber *et al.*, 1989). The concentrations of neutrophil elastase and cathepsin G were also estimated from their extinction coefficients (Travis *et al.*, 1978). Pancreatic and tissue kallikreins as well as plasmin were reconstituted to nominal gravimetric concentrations. The molar concentrations of purified BX9 and secreted APP751 were estimated by quantitative amino acid analysis on an Applied Biosystems 420A analyzer. Active inhibitor concentrations were then determined by titrating the nominal molar concentration with a known concentration of active site-titrated trypsin. Trypsin, EGF-

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[1] The abbreviations used are: APP, β-amyloid precursor protein; EGF-BP, epidermal growth factor-binding protein; NGF, nerve growth factor; mGK, mouse glandular kallikrein; Suc, succinyl; MeOSuc, methoxysuccinyl; Tos, tosyl; pNA, *p*-nitroanilide.

M S K T T K K F N S L C I D L P R D L S L E I Y Q S I A S V
A T G S G D P H S D D F T A I A Y L R D E L L T K H P T L G
S G N D E A T R R T L A I A K L R E A N G D R G Q I N R E G
F L H D K S L S W D L D S V P R V R N R E V C S E Q A E T G
P C R A M I S R W Y F D V T E G K C A P F F Y G G C G G N R
N N F D T E E Y C M A V C G S A I P T T A A S T P D A V D K
Y L D K L G S V D L Q I S S

FIG. 1. **Predicted sequence of the bacterially expressed fusion protein BX9.** The Kunitz domain region (residues 288–345 of APP751) is *underlined*.

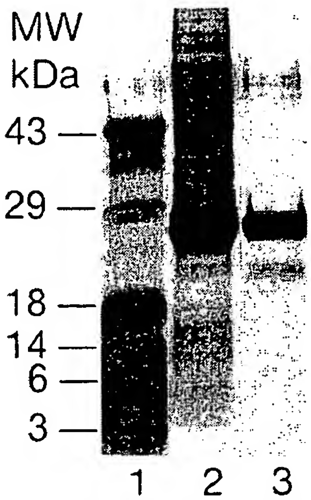

FIG. 2. **SDS-PAGE of BX9.** *Lane 1*, size standards; *lane 2*, crude BX9 (7 M urea extract), approximately 25 μg; *lane 3*, purified BX9, 10 μg.

BP, γ-NGF, mGK-22, pancreatic kallikrein, and plasma kallikrein were all assayed with Pro-Phe-Arg-pNA (S2302), chymotrypsin and cathepsin G with Suc-Ala-Ala-Pro-Phe-pNA, neutrophil elastase with MeOSuc-Ala-Ala-Pro-Val-pNA, thrombin with Tos-Gly-Pro-Arg-pNA, plasmin with Tos-Gly-Pro-Lys-pNA, and tryptase with benzyl-lysine-thiobenzyl ester.

Fractional protease activity remaining, a, was plotted against inhibitor concentration, I_0, and the data were fitted by a nonlinear regression analysis program (Enzfitter, Elsevier) to the equation,

$$a = 1 - \frac{E_0 + I_0 + K_{i(\mathrm{app})} - \{(E_0 + I_0 + K_{i(\mathrm{app})})^2 - 4E_0I_0\}^{1/2}}{2E_0}$$

which led to the direct estimation of $K_{i(\mathrm{app})}$ (Bieth, 1984). K_i values were obtained by correcting for the contribution of substrate K_m by the following equation.

$$K_i = K_{i(\mathrm{app})}/(1 + S/K_m)$$

RESULTS AND DISCUSSION

Fig. 2 shows that the purified fusion protein, BX9, migrated as a ~25-kDa band on reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, in good agreement with its predicted size. The inhibition of pancreatic trypsin (5 nM) by purified BX9 and sAPP751 is shown in Fig. 3. To establish the molar ratio of inhibition, trypsin (100 nM) was titrated with sAPP751 (0–80 nM), and the result is shown in the *inset* of Fig. 3. The linear regression plot gives a molar equivalence point of 0.9:1 (sAPP751:trypsin), indicating that a 1:1 inhibitory complex is formed. (A correction factor of 1.1 was then used to calculate active sAPP751 concentration.) The remarkable level of superimposibility of the two curves in Fig. 3 leads to the calculation of very similar K_i values, 0.2 nM for sAPP751 and 0.3 nM for BX9 (see Table I). The inhibition curves obtained by incubating different proteases (at 25 nM with sAPP751, 30 nM with BX9) with increasing concentrations of sAPP751 (Fig. 4, *top*) or BX9 (Fig. 4, *bottom*) were then used to calculate inhibition constants. The compiled K_i values calculated with both BX9 and sAPP751 are listed in Table I. For either inhibitor, the order of inhibitory potency is trypsin > EGF-BP > α-chymotrypsin > γ-NGF > plasmin > mGK-22. Neither inhibitor appreciably inhibited thrombin, lung tryptase, pancreatic kallikrein, plasma kallikrein, or the granulocyte serine proteases elastase and cathepsin G (K_is ≥ 1 μM).

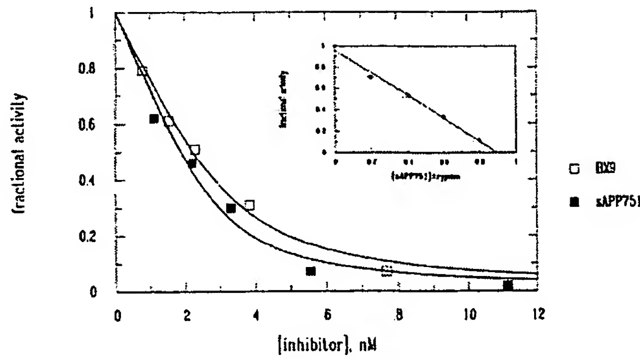

FIG. 3. **Inhibition of trypsin by BX9 and sAPP751.** Trypsin (5 nM) was incubated with 0–12 nM of either BX9 (*open square*) or sAPP751 (*filled square*), and the residual activity was plotted against inhibitor concentration. Curves were fitted and drawn by computer using the Enzfitter program. *Insert*, titration of trypsin (100 nM) by sAPP751 (0–80 nM).

TABLE I

Inhibition constants of BX9 and sAPP751 toward different proteases

Enzyme	Source	K_i	
		BX9	sAPP751
		nM	
Trypsin	Bovine pancreas	0.3	0.2
EGF-BP	Mouse submaxillary	3.5	1.0
α-Chymotrypsin	Bovine pancreas	6.0	3.0
γ-NGF	Mouse submaxillary	9.0	8.0
Plasmin	Human plasma	60	50
mGK-22	Mouse submaxillary	180	180
Elastase	Human granulocytes	NI[a]	NI
Cathepsin G	Human granulocytes	NI	NI
Kallikrein	Porcine pancreas	NI	NI
Kallikrein	Human plasma	NI	NI
Tryptase	Human lung	NI	NI
Thrombin	Bovine plasma	NI	NI

[a] NI (not inhibited) implies a $K_i \geq 1$ μM.

The strong inhibition of pancreatic trypsin by these inhibitors is consistent with the presence of arginine at the P_1 position of the APP Kunitz domain (residue 301 of APP751). EGF-BP and γ-NGF (Taylor *et al.*, 1974; Blaber *et al.*, 1989) are also arginine-specific esterases, and their relatively strong inhibition by the Kunitz domain is therefore not unexpected. Surprisingly, mGK-22, which is closely related to both of these proteases (Drinkwater *et al.*, 1987) and was earlier throught to be an epidermal growth factor-binding protein (Anundi *et al.*, 1982; Blaber *et al.*, 1987), is rather poorly inhibited (K_i ~ 180 nM), suggesting that specific interactions other than P_1–S_1 must play an important role in determining inhibitor potency. The interaction with α-chymotrypsin (which does not have affinity for basic residues at P_1) is also likely explained by favorable non-P_1–S_1 interactions. It should be noted that α-chymotrypsin is also inhibited (K_i = 6 nM) by bovine pancreatic trypsin inhibitor (Fritz and Wunderer, 1983), which is otherwise selective for trypsin-like proteases.

The poor inhibition of plasmin and the lack of inhibition

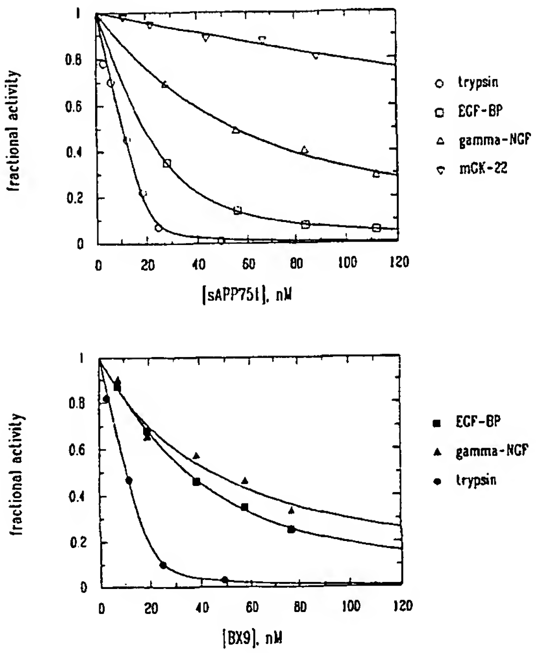

FIG. 4. **Inhibition of proteases by sAPP751 (*top*) or BX9 (*bottom*).** 25 nM (with sAPP751) or 30 nM (with BX9) trypsin (*circle*), EGF-BP (*square*), γNGF (*triangle*), and mGK-22 (*inverted triangle*) were incubated with 0-120 nM sAPP751 or BX9, and the residual activities were plotted against inhibitor concentration. Curves were fitted and drawn by computer using the Enzfitter program.

of thrombin, tryptase, and pancreatic and plasma kallikreins by the APP Kunitz domain suggest that this inhibitor has restricted selectivity even for proteases with trypsin-like specificity. With the exception of that for pancreatic trypsin, the K_i values do not suggest a very potent inhibitor but indicate that potential target proteases will likely be arginine-specific esterases. A human homolog of either EGF-BP or γ-NGF has not yet been reported, and, in fact, the human kallikrein gene family seems to have far fewer members (Schedlich *et al.*, 1987) than the mouse kallikrein family to which these curious arginine esterases belong. It is thus difficult to predict what the identity of the "physiologically relevant" protease, with which the APP presumably interacts, might be. Another possibility, of course, is that a true trypsin-like protease is expressed in the same tissues, including brain, where the APP is expressed.

The bacterially expressed fusion protein inhibitors, BX9 and sAPP751, have identical qualitative inhibitory profiles toward all the proteases tested in this study and, in addition, generate very similar K_i values with the proteases examined. The protease inhibitory properties of the APP thus appear to reside entirely in its Kunitz domain, suggesting other functions for the remaining domains of this large and complex protein. It is not clear whether these inhibitory properties are related to the deposition or pathology of the β-peptide, which is apparently proteolytically excised from the APP. However, the identification of a specific protease in human brain which physiologically interacts with the APP Kunitz domain could shed more light on the pathogenesis of Alzheimer's disease.

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